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**Short communication: Immunoglobulin variation in quarter milked colostrum**

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Running Head: Quarter colostrum

Interpretive Summary

The process of colostrum formation in dairy cows has been shown to be highly variable resulting in first milked colostrum of differing quality. Quarter milking of dairy cows within 3 h of parturition shows that each independent quarter can be significantly different in concentration and mass of IgG1. This finding helps to explain the vast variation in total first milked colostrum IgG1 concentration and mass in that each cow may have any number of quarters that produce high quality colostrum, the inverse situation, or any other combination. While this explains some of the colostrum variation, it creates another question. How can each quarter be independent in colostrum formation when each gland has the same DNA and is exposed to the same concentrations of circulating endocrine factors that have been attributed to the development of colostrogenesis?

## ABSTRACT

While whole first milked colostrum IgG1 variation is documented, the IgG1 difference between the quarter mammary glands of dairy animals is unknown. First colostrum was quarter collected from healthy udders of 8 multiparous dairy cows, all within 3 h of parturition. Weight of colostrum produced by individual quarters was determined and a sample of each was frozen for subsequent analysis. IgG1 concentration (mg/mL) was measured by ELISA and total mass (g) was calculated. Standard addition method was utilized to overcome colostrum matrix effects and validate the standard ELISA measures. Analysis of the data showed that cow and quarter(cow) were significantly different in both concentration and total mass per quarter. Analysis of the mean IgG1 concentration of the front and rear quarters showed that this was not different, but the large variation in individual quarters confounds the analysis. This quarter difference finding indicates that each mammary gland develops a different capacity to accumulate pre-colostrum IgG1, while the circulating hormone concentrations that induce colostrogenesis reach the 4 glands similarly. This finding also shows that the variation in quarter colostrum production is a contributor to the vast variation in first milking colostrum IgG1 content. Finally, the data suggests other factors such as locally acting autocrine/paracrine, epigenetic, or stochasticity in gene regulation mechanisms may impinge on colostrogenesis capacity.

## INTRODUCTION

The importance of adequate passive transfer (APT) of colostrum immunoglobulin to the newborn for minimizing morbidity and mortality has been demonstrated in multiple studies (Donovan et al., 1998; Quigley, III et al., 1995; Weaver et al., 2000). Although the health benefits of APT are clear, the reality of calf rearing is that a high proportion of calves on ranches and dairies are deprived of an adequate mass of immunoglobulins leading to failure of passive transfer (FPT) (Gay et al., 1983; Lorenz et al., 2011). Calves with APT immunity have lower mortality and morbidity and fewer antibiotic treatments compared with animals with FPT (Berge et al., 2005).

Failure of passive transfer is reported to be 19% in US dairy heifer calves (Beam et al, 2009) and part of this problem is attributed to feeding colostrum with a low immunoglobulin concentration (<50 mg/mL of IgG) or feeding an inadequate volume of colostrum with low colostrum IgG concentration (Weaver et al., 2000; Godden, 2008). The concentrations of IgG1 and IgG2 in serum of dairy cows are approximately equal at ~5 to 12 mg/mL, but colostrum shows a very selective mammary transfer of IgG1 accounting for up to a ~10-fold enhancement (Sordillo et al., 1987). However, cow colostrum has extremely high animal to animal variation in IgG<sub>1</sub> concentration (Quigley et al., 2001; Morin et al., 1997 ). Kehoe et al. (2007) showed IgG concentration varying from 11.8 to 74.2 mg/mL and Baumrucker et al. (2010) described the mass variation [30g to >2Kg]. The current explanation of this variation could be any number of factors including endocrine (Casey and Plaut, 2007), genetic (Doleschall et al., 2005; Mayer et al., 2005), lactation number, breed of cow, length of the non-lactating period (Muller and Ellinger, 1981; Pritchett et al., 1991; Tomkins and Jaster, 1991 Mansfeld et al., 2012) and the method of analysis (Li-Chan and Kummer, 1997).

Still another source of the variation has been attributed to the time of first milking following parturition. Copious quantities of mature milk components appear en masse following parturition and with delayed colostrum milking and the inclusion of osmotic

68 molecules such as lactose and ions begin to facilitate more water that has a diluting effect on  
69 IgG1 concentration. Thus, such water inclusion induces potential variation in each animal  
70 colostrum sample when measured as concentration, but would not be expected to affect  
71 colostrum total mass of IgG1. Thus, we have affirmed that total mass of IgG1 transferred by  
72 a mammary gland would be independent of water influx and more useful in establishing  
73 animal variation in transport capacity and perhaps transport mechanisms that are present  
74 during dairy cow colostrogenesis (Baumrucker et al., 2010).

75 While total colostrum IgG variation in concentration and mass is recognized, the  
76 variation within animal quarter is not established. Quarter samples of immunoglobulins taken  
77 on the same day pre- and post parturition were reported to vary widely (Kiddy et al., 1971),  
78 but no values were provided. We hypothesized that quarter mass production of IgG1 within a  
79 cow would not be different. The objective of this study was to 1) establish and validate an  
80 ELISA method for analysis of colostrum IgG and 2) determine the udder quarter variation of  
81 IgG1 concentration and mass in first milked colostrum.

## 83 MATERIALS AND METHODS

### 84 *Animals*

85 Eight dairy cows of Holstein Friesian and Swiss Fleckvieh breed with approximately  
86 80% Red Holstein genes were evaluated. All cows were in their second or third parity from  
87 the Agroscope Liebefeld-Posieux (ALP) research station in Switzerland and were milked  
88 within 3 hours of parturition without calf suckling. All quarters were free of mastitis events  
89 during the collection period. In the prescribed experiment each quarter's milk volume was  
90 completely collected in a separate bucket by a quarter milking machine based on a Surge RX  
91 quarter milking claw (GEA, Böhnen, Germany). The machine teat-cups were applied and  
92 withdrawn by hand. Each bucket was weighed separately and recorded and a 50ml aliquot

was stored at -20°C until subsequent analysis. Mastitis treatment by quarter(cow) were obtained from the ALP Station veterinary records.

### ***IgG1 analysis***

IgG1 was measured by an ELISA according to manufacturer's protocol (ELISA Quantitation Set; Bethyl Laboratories Inc., LuBioScience, Luzern, Switzerland), except that the blocking solution was exchanged with a solution made out of bidistilled water and fish gelatin (1ml of cold water fish skin gelatin (G7765; Sigma Aldrich, Steinheim, D) per 20ml of bidistilled water). The colostrum fractions for this assay were diluted in 1:10 sequential series with the final in plate dilutions of  $1 \times 10^5$ ,  $4 \times 10^5$ ,  $8 \times 10^5$ , and  $1.6 \times 10^6$ . This was performed because the range of IgG1-content is extremely variable between animals and extensive dilutions are required to overcome matrix effects. The described dilutions of the colostrum were assayed and a mean and SD was obtained that reflected in-plate analysis error. Sample analysis was considered acceptable if in-plate dilutions resulted in concentrations with CV of <12%. If the CV was exceeded, the samples were re-analyzed with suitable dilutions. Inter-assay repeatability was <16% CV.

### ***Standard Addition Method***

The assay of complex mixtures of components, such as bovine colostrum, can be a problem with interpolating from a standard curve because the unknown sample may contain other substances that interfere with the assay. Dilutions of a colostrum sample determined by an ELISA assay, such as the Bethyl IgG assay described above, will result in increasing concentration of IgG with increasing dilution due to the reduction of interfering substances. This is known as the "matrix effect problem". However, at high dilutions, the matrix effect is reduced or eliminated and the diluted samples come to equivalency. The Standard Addition Method (SAM) is a way to overcome this problem. In the SAM procedure, the unknown

concentrations are not established by a separate standard curve. Instead, various known concentrations (including zero) of the standard are added to a constant amount of diluted unknown. This ensures that all the samples have the same amount of unknown, including any substances that interfere with the assay.

In order to test the matrix effect of bovine colostrum, we utilized the standard addition method to circumvent matrix effects and validate our procedure for the ELISA dilution assay. The Standard addition method requires a linear analytical response to dilutions of the sample. However, the typical ELISA standard curve for IgG1 in colostrum is a sigmoid curve. Nevertheless, the lower range of the IgG1 standard curve is linear (lower 3-4 values). Therefore, we utilized this linear component of colostrum analysis and conducted the standard addition method with colostrum samples of high to mid concentrations of IgG1 that was established by the ELISA analysis described above. Figure 1 shows the results of this method with one sample of a relatively high concentration sample of IgG1 that was determined by ELISA described above to be a mean and SD of  $96.3 \pm 9.5$  mg/mL of IgG1 with a CV of 8.8%. Figure 1 shows the relatively high IgG1 concentration sample with extrapolated concentrations adjusted for dilution of 93.4, 96.0, 90.8, and 94.7, mg/mL for the four dilutions of colostrum. The standard extrapolates to zero. This analysis resulted in a mean of  $93.7 \pm 2.1$  mg/mL of IgG1 with a CV of 2.3%. The colostrum medium range concentration sample produced similar results with a CV of 6.8% when compared to the ELISA assay results (data not shown).

### ***Statistics***

Immunoglobulin G<sub>1</sub> mass and concentration established by the ELISA assay were averaged across the four dilutions for each quarter of each cow. This resulted in 32 total observations for IgG1 mass and concentration which were then evaluated with the MIXED

procedure of SAS (SAS Institute Inc., Cary, NC). Cow was included as a fixed effect with the denominator degrees of freedom determined using the Satterthwaite approximation (ddfm=sat) for significance testing. A REPEATED statement with the GROUP option set to cow was included to fit a separate residual variance for each cow. Akaike's information criterion (AIC), corrected AIC (AICC), and Bayesian information criterion (BIC) were then compared among models with and without the REPEATED line to evaluate whether there was significant evidence of heterogeneous variance among cows. Attempts to fit mixed models that included effects for both cow and udder half (front versus rear) did not converge. Therefore, an ANOVA model that included the fixed effects of cow, half, and half within cow was evaluated using the GLM procedure of SAS. This model assumed homogenous variance across all cows.

## RESULTS

Utilization of the SAM (Figure 1) for quantification of IgG1 concentration in first milked colostrum showed a mean value of 93.4 mg/mL for the 5 diluted samples with a CV of 2%.. This mean value agrees with the ELISA values of 96.3 mg/mL for the 4 in-plate dilutions conducted on the same sample. This finding confirms that extensive dilution of the colostrum sample reduces the matrix effect of colostrum in the standard ELISA assay as described in our methods so as to reach an accurate estimate of IgG1 concentration. Such dilutions may exceed  $1 \times 10^6$  for highly concentrated IgG colostrum samples.

Both the mixed and ANOVA models confirmed that cow had a significant effect on IgG1 mass and concentration. Additionally, fitting a heterogeneous variance structure improved fit statistics (AIC, AICC, BIC) for the evaluation for IgG1 concentration which indicated that the amount of variation among quarters was different among cows. Fitting a heterogeneous variance structure also improved the model fit for IgG1 mass according to AIC and AICC, but not for BIC. Figure 2 shows the results of IgG1 concentration and mass



collected by quarter from eight individual cows. The columns correspond to the average of the four dilutions for IgG1 mass or concentration with lines that indicate the standard deviation among the dilutions. The relatively small lines demonstrate that the dilutions resulted in repeatable results for each cow. Within each animal figure is printed the residual (R) from the heterogeneous variance analysis. Figure 2 indicates that IgG1 concentration varied minimally among quarters for four cows (1601, 1609, 1627, and 1695) as indicated by the low R. Also, IgG1 mass varied minimally among quarters for cows 1601, 1609, and 1695 as shown by their low R. It is notable that 1627 mass R is high while the concentration R is low.

Figure 2 also indicates that IgG1 concentration was highly varied among quarters for cows 1296, 1530, and 1685, as shown by their high R. Similarly, IgG1 mass was highly varied among quarters for cows 1296, 1530, 1627, 1640, and 1685 as indicated by their high R.

Finally, we tested front against rear quarters for differences in concentration and mass of IgG1. There were no significant differences between udder halves for the ANOVA analyses. Figure 3 shows the variation between front and rear quarters of the experimental cows. The LSMEANS for IgG1 concentration were 52.4 and 46.1 mg/mL for front and rear udder halves, respectively. The mass of IgG1 exhibited LSMEANS of 86.5 and 104.6 g, front and rear udder halves, respectively. The analysis indicated that cow ( $P=0.0003$ ) was significant while udder half ( $P=0.295$ ) and udder half within cow ( $P=.6507$ ) was not significant. While 3 cows (1296; 1640, 1685) show large mean differences in mass of IgG1 between the front and rear quarters, the quarter variation shown in Figure 2 is very large so as to produce a statistical test that is non-significant.

## Discussion

The use of the standard addition method allows for the control of matrix effects if the analytical output produces a linear result. While most ELISA results are sigmoid curves, the lower range of detection is linear. We used these values to show that the standard addition method applied to highly diluted bovine first milk colostrum results in a concentration that was equivalent to our standard ELISA assay. These results validate the use of the ELISA we describe in the methods to quantify IgG1 in bovine colostrum.

The concentration and mass of IgG1 in first colostrum of cows has been shown to be vastly different between cows and not related to the mass of parenchyma tissue (Baumrucker et al., 2010; Kehoe et al., 2007). A recent report by Kessler et al (2013) provides more evidence of the disconnect between mature milk secretion volume and colostrum volume. Furthermore, while concentration and mass of IgG1 in first milked colostrum are related ( $R^2 \sim 0.65$ ; Baumrucker et al., 2010) within an animal, they can be significantly different. An animal that has higher concentration is more likely to produce greater mass, but a lower concentration colostrum can achieve greater mass by the production of high volume (mass=concentration x volume). One explanation for the concentration difference between animals is the variation in the timing of the beginning of differentiation from colostrum formation and that of copious mature milk production. This process has been described as the change from Lactogenesis I (proliferation, differentiation and colostrogenesis) to Lactogenesis II (nature milk production) (Nguyen and Neville, 1998) and begins to occur days before parturition. Each cow may begin the process at different times that results in the formation of mature milk production that includes more lactose and ions resulting in increased water content added to the preformed colostrum thereby diluting IgG1 concentration at first milking. Furthermore, if quarters develop at different rates from Lactogenesis I into the Lactogenesis II stage, then concentration differences could be explained. Nevertheless, there is no current data to support mammary quarter differential development within an animal.

While this explanation may explain concentration variation between the cow mammary gland quarters, water inclusion has little or no effect upon total mass produced by a quarter of the mammary gland. It was therefore surprising to find that quarter(cow) mass was different. We therefore reject our initial hypothesis that quarter mass production of IgG1 within a cow would not be different.

Different quarters are known to produce different volumes of milk during an established lactation with the rear quarters generally producing more milk volume due to the greater mass of parenchyma tissue (Tucker, 1981). In our analysis, the front quarters produced colostrum with higher IgG1 concentration when compared with the rear quarters (52>46 mg/mL). This may be explained by the greater dilution effect occurring by secretory activation (Lactogenesis II) in these rear quarters with more secretory tissue. In support of this idea, mass was greater in the rear quarters (104>86) suggesting the rear location has some relevance to IgG1 transcytosis. However, referring back to Figure 2, it is clear that the averaging of some quarters with extreme differences (variation) to produce a mean for an udder half does not provide a clear answer to the question of IgG1 production.

Anatomical studies have clearly shown that a cows` udder is divided into 4 separate quarters (Hammond J., 1927). Many experimental paradigms have utilized this knowledge to test one quarter while utilizing another for a control (i.e.: mastitis experiments with lipopolysaccharide). It is also known that the steroid hormones released from the conceptus during pregnancy and involved in the initiation of colostrogenesis result in increases in the blood concentrations and that each separate quarter of the cow mammary gland would be exposed to very similar concentration of these hormones (Guy et al., 1994). Our findings show that any quarter within a cow udder may produce different concentrations and mass of IgG1 in the first milked colostrum that appears to be unrelated to parenchyma tissue mass and is supported by previous findings with total colostrum (Baumrucker et al., 2010). An extremely low total

mass production in first milked colostrum of any cow would clearly indicate that all quarters are rather poor in the transcytosis of IgG1 from the blood to the secretions while an extremely high quality colostrum producer would likely have all quarters functioning extremely well in IgG1 mass transfer. Those animals with average transfer of mass could be illustrated by cow 1685 (Figure 2). Clearly, the quarter variation in colostrum formation is a major contributor of the variation in first milked colostrum IgG1 content (Baumrucker et al., 2010).

It is known that while each mammary gland is independent, the lactating tissue is developed from the same general copies of DNA from pre-existing mammary epithelial cells. Previous lactation cell numbers that remain for the subsequent lactation may be contributors to this variation. Such variation may be resolved by testing first lactation animals and we are in the process of such experiments. However, genetic contribution to this quarter variation cannot be explained unless epigenetic mechanisms (Rijnkels et al., 2013) are contributing to colostrogenesis and are influenced independently within each quarter. Perhaps, stochasticity in gene expression (Koern et al., 2005) may also be included in the list of unknown regulatory factors. Still another explanation may be the effect of differential expression of local factors, paracrine or autocrine, that contribute to the colostrogenesis mechanisms induced by steroids (Guy et al., 1994). However, quarter differential expression of these and other factors within the same animal is currently undocumented. It is also possible that previous mammary quarter disease problems (mastitis) contribute to this variation. The latter explanation may account for necrotic damage to mammary epithelium and could also result in independent epigenetic control of separate quarters. However, necrotic damage would lead to less productive parenchymal tissue and our previous work suggested that colostrum production was not related to parenchymal tissue mass (Baumrucker et al., 2010) and a new report (Kessler et al., 2014) indicates no relationship between colostrums volume and subsequent milk volume within an animal. We had limited quarter disease information for the cows described in this

265 research and our sample size was too limiting, thus no conclusion could be reached. More  
266 research is needed to shed light on the factors that control this process.

267 Finally, these findings about colostrogenesis in the mammary glands of cows indicate  
268 that the sampling of one mammary gland fluid or tissue for analysis of colostrogenesis may  
269 not reflect the process occurring in the other quarters of the udder. It would be interesting to  
270 know if this is also occurring in other species of mammals.

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272

## 273 Figure Legends

274

275 Figure 1. Standard addition method plot utilizing a first milked colostrum sample. Our  
276 ELISA procedure (methods) quantified the IgG1 as  $96.3 \pm 8.5$  mg/mL. The Y axis is not  
277 shown but is typically the response factor (absorption in plate reader). Extrapolation to the x-  
278 axis resulted in the negative values that represent the positive concentration of the diluted  
279 sample. When each dilution factor was calculated with the X value it produced the mean of  
280  $93.4 \pm 2.1$  for the 5 diluted samples.

281

282 Figure 2. IgG1colostrum concentration and mass in first milked quarters.

283 Data is the mean of concentration and mass of IgG1 for individual cows and their quarters.  
284 Also shown for each quarter is the residual (R) from the heterogeneous model described in  
285 methods and materials. The error bars indicate the SD of for each quarter among in-plate  
286 dilutions.

287

288 Figure 3. IgG1colostrum concentration and mass in first milked front and rear quarters.

289 Data is the mean  $\pm$  the SD of either concentration or mass of IgG1 for each cow front and rear  
290 quarters. Analysis indicated that cow ( $P=0.0003$ ) was significant while udder half ( $P=0.295$ )  
291 and udder half within cow ( $P=.6507$ ) were non-significant.

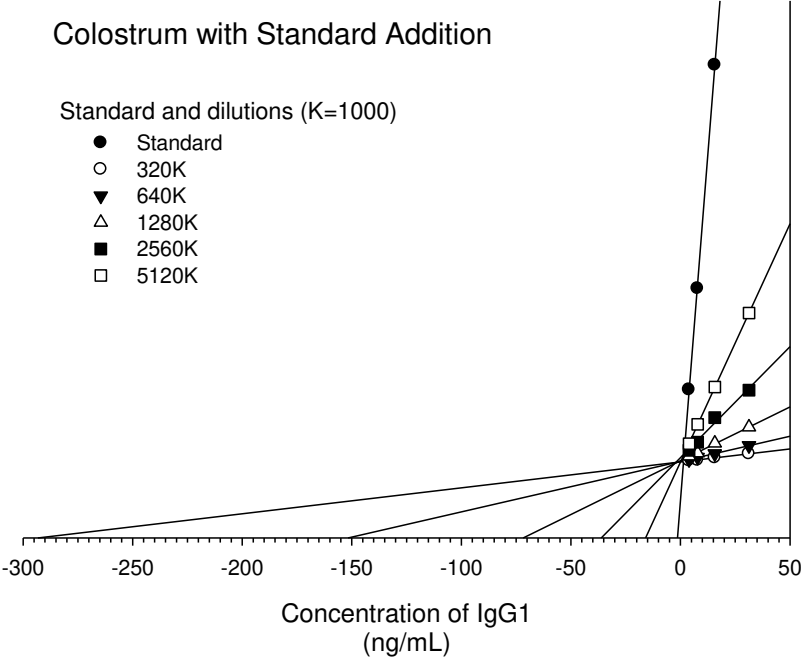
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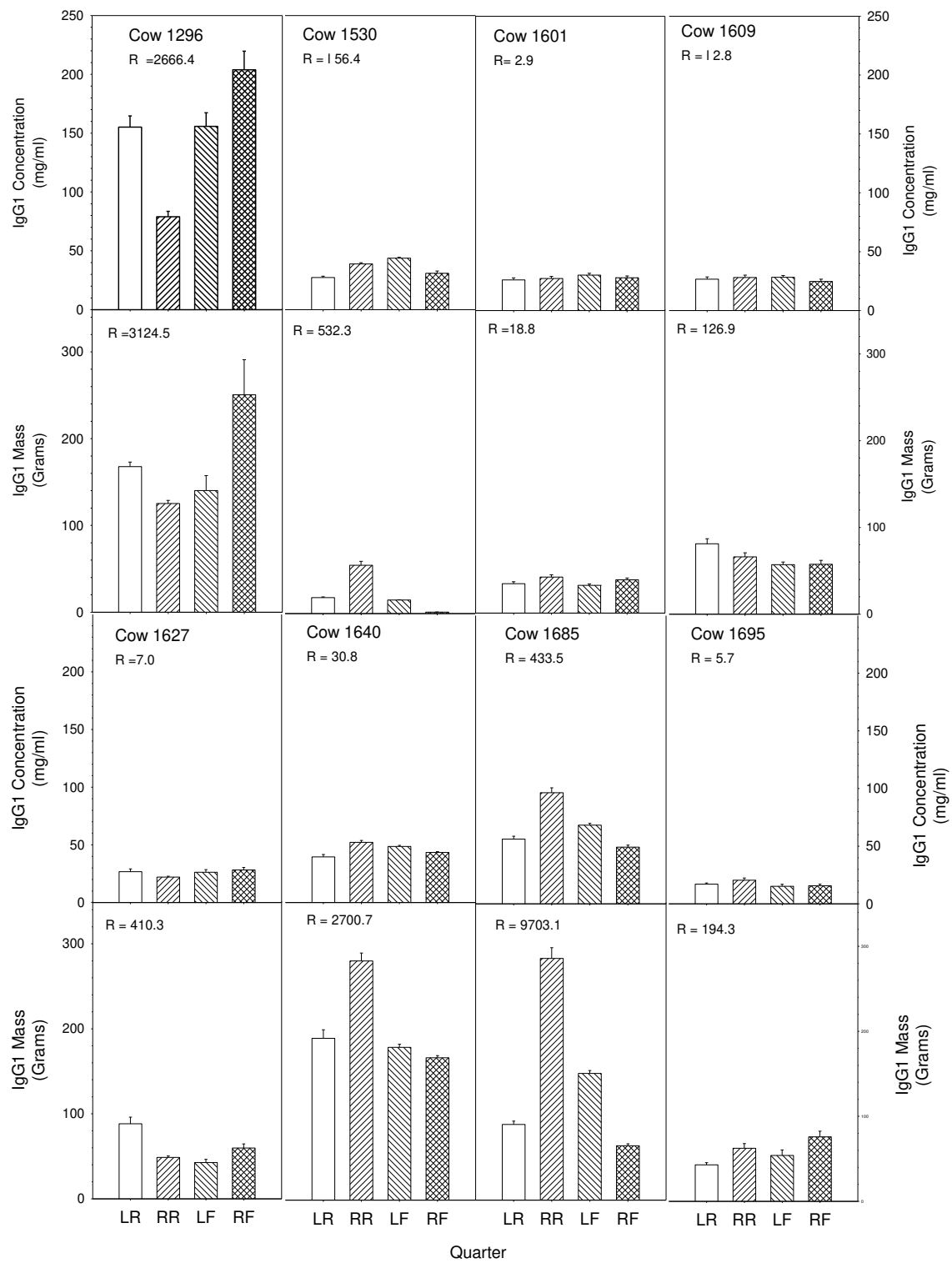
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Figure 2 Baumrucker et al

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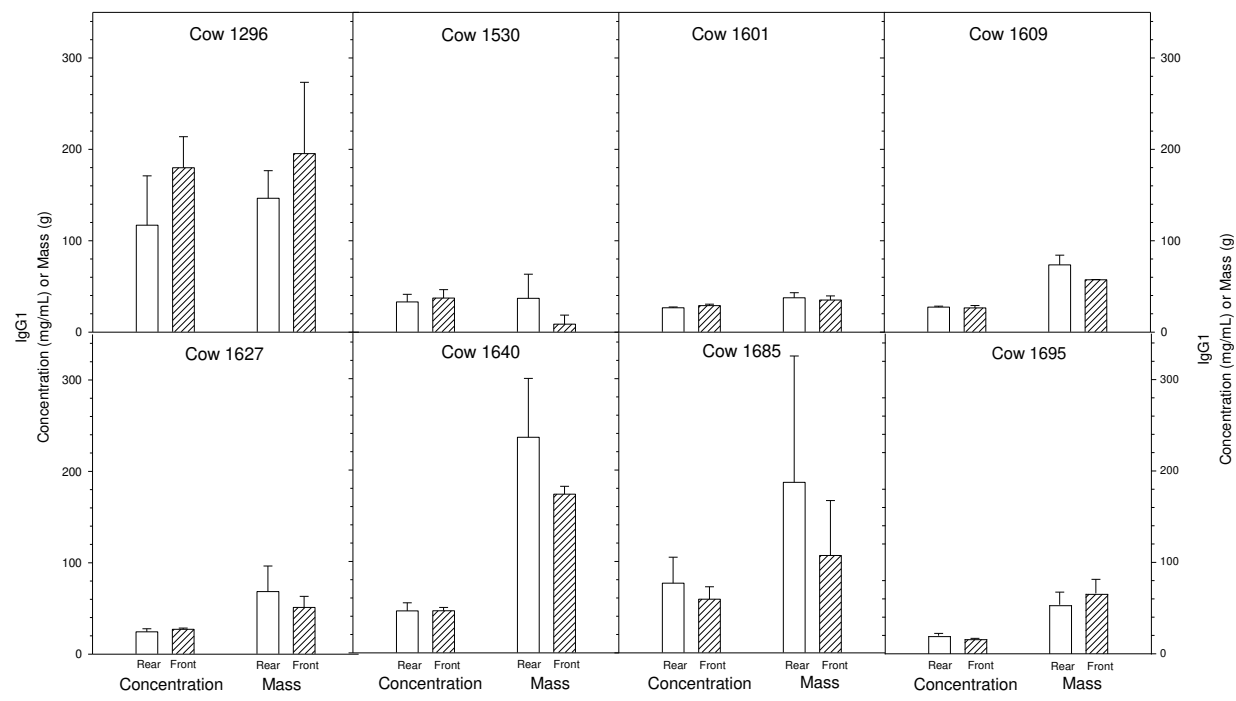
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Figure 3 Baumrucker et al. 2013

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